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Effects of triclocarban on the transcription of estrogen, androgen and aryl hydrocarbon receptor responsive genes in human breast cancer cells

Patrick Tarnow *, Tewes Tralau, Danele Hunecke, Andreas Luch

German Federal Institute for Risk Assessment (BfR), Department of Product Safety, Berlin, Germany

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ABSTRACT

Triclocarban (TCC) is an antimicrobial agent that is used in detergents, soaps and other personal hygiene products. Similarly to triclosan the widespread use of TCC has raised concerns about its endocrine potential. In luciferase-based reporter assays TCC has been shown to enhance estrogenic and androgenic activities following cellular coexposure with estrogen or dihydrotestosterone, respectively. The present study demonstrates that although coexposure with TCC enhances the estrogenic and androgenic readout of luciferase-based reporter cell lines such as HeLa9908 and MDA-kb2, it fails to act as a xenoandrogen on transcriptional level, nor does it induce cell proliferation in the estrogen sensitive E-screen. In addition TCC did not alter the expression of estrogen responsive genes in human mammary carcinoma MCF-7 cells exposed to 17β-estradiol, bisphenol A, butylparaben or genistein.

However, TCC was shown to interfere with the regulon of the aryl hydrocarbon receptor (AhR) as TCC showed a costimulatory effect on transcription of *CYP1A1* and *CYP1B1*, effectively lowering the transcriptional threshold for both genes in the presence of estrogens. It thus seems, that while the induction of the respective luciferase reporter assays by TCC is an unspecific false positive signal caused by luciferase stabilisation, TCC has the potential to interfere with the regulatory crosstalk of the estrogen receptor (ER) and the AhR regulon.

1. Introduction

Triclocarban (3,4,4′-trichlorocarbanilide, TCC) is an antimicrobial agent commonly added to detergents and personal hygiene products including liquid soaps or soap bars. Apart from its diphenylurea moiety TCC is structurally similar to other widely used antimicrobials such as triclosan (TCS) and hexachlorophene (HCP) (Fig. 1). The use in soaps results in direct human exposure. Liquid soaps contain up to 1.5% of TCC (SCCP, 2005) and for a single shower the absorption of TCC is estimated to be 0.6% (Schebb et al., 2011). Based on an average use of 20 g of soap per shower TCC can therefore be expected to reach concentrations of approximately 1 μM in the blood stream. This was recently confirmed in a study with human volunteers, where the use of TCC containing soap resulted in half-maximal blood concentrations of up to 530 nM (Schebb et al., 2012). Moreover, in the US its ubiquitous use has led to concentrations as high as 6.8 μg/l in environmental water samples (Halden and Paull, 2005). As a halogenated hydrocarbon TCC is hardly biodegradable (Aken et al., 2010; Furukawa and Fujihara, 2008; Solyanikova and Golovleva, 2004) and subsequent levels in sewage sludge easily exceed 50 mg/kg (Heidler et al., 2006). In combination with the frequent use of sewage as fertiliser the poor biodegradability thus further adds to human exposure (Wu et al., 2012).

The high levels of TCC in water and sewage have raised concerns because TCC has been shown to amplify estrogenic and androgenic responses in cell-based reporter assays (Ahn et al., 2008). Androgenic effects were also observed *in vivo*. In castrated rats the coadministration of TCC and testosterone resulted in higher weights of sex accessory organs (Chen et al., 2008). Respective hyperplasias were also found in juvenile animals after they had been treated with TCC (Duleba et al., 2011). Meanwhile the estrogenic effects of TCC *in vivo* are less well investigated. In zebrafish coexposure to 17β-estradiol (E2) and TCC enhanced the transcriptional

Abbreviations: AhR, arylhydrocarbon receptor; AP-1, activator protein; AR, androgen receptor; ATP, adenosine triphosphate; BPA, bisphenol A; BuPa, butylparabene; *CCND1*, cyclinD1, CYP, cytchrome P450; DHT, dihydrotestosterone; E2, 17β-estradiol; EC_{50}, half maximal effective concentration; ER, estrogen receptor; ERα, estrogen receptor alpha; ERβ, estrogen receptor beta; ERE, estrogen responsive element; *ESR1*, estrogen receptor alpha; *ESR2*, estrogen receptor beta; Gen, genistein; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *HSPB8*, heat shock binding protein 8; *NDRG1*, N-myc downstream regulated 1; *PGR*, progesterone receptor; *RPLP0*, ribosomal protein, large, P0; *SARG*, specifically androgen-regulated gene protein; SEM, standard error of the mean; *SORD*, sorbitol dehydrogenase; Sp1, specificity protein; TCC, Triclocarban; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; *TFF1*, trefoil factor 1; *UGT2B15*, glucuronosyltransferase 2B15.

* Corresponding author. Address: German Federal Institute for Risk Assessment (BfR), Max-Dohrn Strasse 8-10, 10589 Berlin, Germany. Tel.: +49 30 18412 3885.
E-mail address: Patrick.Tarnow@bfr.bund.de (P. Tarnow).

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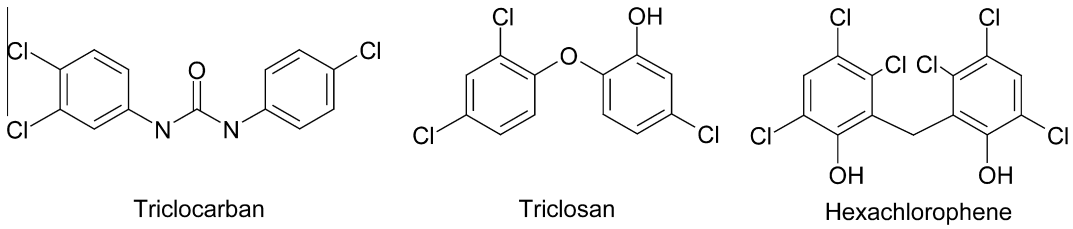

Fig. 1. Chemical structures of the antimicrobials triclocarban (TCC), triclosan (TCS) and hexachlorophene (HCP).

induction of aromatase AroB, while the combination of TCC with the xenoestrogen bisphenol A (BPA) led to reduced expression of *aroB* (Chung et al., 2011).

Estrogens exert their effects mainly via two nuclear receptors, that is estrogen receptor alpha (ERα) and beta (ERβ). Following cognate ligand binding these transcription factors dimerise and bind to specific estrogen response elements (EREs) at the DNA, where subsequent recruitment of co-activators induces target gene expression (Heldring et al., 2007). Alternatively ERs can interact with transcription factors already bound to the DNA, such as AP-1 and Sp1. A third mechanism is the activation of non-genomic pathways, where hormone binding leads to the rapid activation of signalling cascades (Heldring et al., 2007).

Most estrogenic reporter gene assays use ERE-containing promoters in combination with endogenous or transgenic ERα. Nevertheless, several estrogen responsive genes do not contain classical EREs. Instead these promotors contain ERE half-sites, AP-1- and Sp1-sites or combinations thereof (O'Lone et al., 2004). This suggests the regulation of endogenous genes to be more complex and questions the suitability of assays with readouts that are solely based on ERE-driven gene expression. Therefore this study aimed to compare the results of commonly used reporter gene assays with the effects of TCC on endogenous gene expression in human mammary carcinoma cells. The examined transcripts include androgenic and estrogenic target genes as well as genes of the AhR regulon. Androgenic gene expression was examined in an ER^- background (i.e. MDA-MD-453), while MCF-7 cells were used to test the influence of TCC in combination with E2 and a choice of xenoestrogens typically found in consumer products, cosmetics and foods (Evans et al., 2012).

2. Materials and methods

2.1. Chemicals

Cell culture media were purchased from PAN Biotech (Aidenbach, Germany), charcoal treated FCS was obtained from PAA (Cölbe, Germany) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was a gift from the German dioxin reference lab (BfR, Berlin, Germany). Substrates for the luciferase assays (D-Luciferin, ATP) and reducing agent DTT were obtained from PJK (Kleinblittersdorf, Germany). All other chemicals were purchased from Sigma Aldrich (Munich, Germany). Substances were routinely dissolved in ethanol, with the exception of TCDD and TCC for which dimethylsulfoxide (DMSO) was used.

2.2. Androgen reporter assay

Cell line MDA-kb2 was obtained from the ATCC (ATCC-No. CRL-2713). The MDA-kb2 cell line is a derivative of MDA-MD-453 breast cancer cells. The latter provide a well characterised molecular background for androgenic testing, as they express the androgen receptor (AR) but are negative for ER. Transfection of this cell line with a stable MMTV.luciferase.neo reporter gene construct yielded the MDA-kb2 reporter cell line which is responsive to stimulation of the AR and the glucocorticoid receptor (GR) (Wilson et al., 2002). Upon arrival in the lab cellular transcription of the AR was confirmed by quantitative RT-PCR, as was the absence of transcripts for ER (Fig. S1). Reporter assays were performed as described by Ermler et al. (2010). Briefly, MDA-kb2 cells were maintained in Leibowitz' L-15 medium supplemented with FCS (10% v/v) and grown at 37 °C without the provision of additional CO_2. A week before usage the cells were switched to phenol red free L-15 medium with charcoal treated FCS (5% v/v). Subsequent seeding into 96-well plates was done one day prior to exposure, using a concentration of 10^4 cells per 100 µl and well. Substance exposure was started after 24 h by adding 50 µl of concentrated substance stocks. Dose response curves were measured in triplicate, and controls (1 nM dihydrotestosterone (DHT) and 0.1% ethanol, respectively) were repeated 6-fold.

Measurement of luciferase activity was performed in cellular crude extracts using a Synergy HT plate reader from BioTek (Bad Friedrichshall, Germany). Cells were lysed *in situ* using 50 µl of lysis buffer (0.1 M tris–acetate, 2 mM EDTA, and 1% triton-x, pH 7.8), shaking the plate moderately for 20 min at room temperature. Following cellular lysis 150 µl of luciferase buffer (25 mM glycylglycine, 15 mM $MgCl_2$ and 4 mM EGTA, 1 mM DTT, 1 mM ATP, pH 7.8) and 50 µl of luciferin solution (25 mM glycylglycine, 15 mM $MgCl_2$ and 4 mM EGTA, 0.2 mM luciferin, pH 7.8) were added automatically to each well in order to measure luminescence. All values were corrected for the mean of the negative control and then related to the positive control which was set to 100%.

2.3. Estrogen reporter gene assay

Cell line HeLa9903 was obtained from the JCRB (JCRB-No. 1318). These cells contain stable expression constructs for human ERα and firefly luciferase, respectively. The latter is under transcriptional control of five ERE promoter elements from the vitellogenin gene. The transcription of ERα was confirmed by RT-PCR, as was the absence of AR-transcripts (Fig. S1). The assay was performed according to the OECD test guideline TG455 (OECD, 2009) as follows. Cells were cultivated in phenol red free MEM containing 10% (v/v) of charcoal stripped FCS at 37 °C in an atmosphere with 5% CO_2. For the actual assay cells were seeded into white 96-well polystyrene plates at a concentration of 10^4 cells per 100 µl and well (Costar/Corning, Amsterdam, Netherlands). Test substances were added 3 h after seeding by adding 50 µl of triple concentrated substance stocks to each well. As before dose response curves for treated samples were measured in triplicate, while controls (1 nM E2 or 0.1% ethanol, respectively) were repeated 6-fold. After 24 h of stimulation, cells were washed with PBS and then lysed using 50 µl of lysis buffer and moderate shaking for 20 min at room temperature. Subsequent measurement of luciferase activity was performed analogous to the aforedescribed androgen reporter gene assay. All values were corrected for the mean of the negative controls and then related to the positive controls set as 100%.